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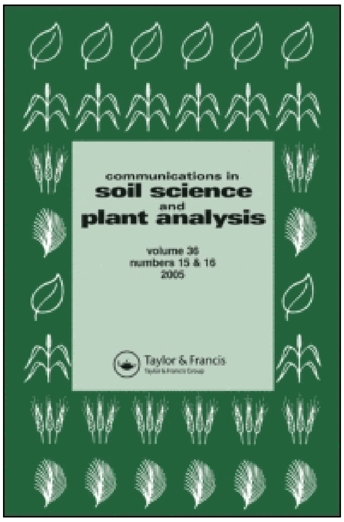

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Determinants and Prediction of Carbon/Nitrogen Ratio in Five Diverse Crop Plants

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Abstract: Multivariate relationships in and statistical moments of eight biochemical constituents and their impact on estimating carbon/nitrogen (C/N) ratio in alfalfa, corn, soybean, cuphea, and switchgrass residues indicate that (1) equal portions of variation in C/N were explained by differences among crops and among organs; however, the largest variations in N and C were explained by differences among crops and among organs within crops, respectively; (2) variation in N, but not in C or N + C, content explained the greatest variance in C/N ratios; (3) biochemically, stems were closer to roots than to leaves; hence the large portion of variation in C/N ratio in roots explained by variation in biochemical constituents in stems and leaves (R^2 = 61.0%) and in stems only (R^2 = 58.0%); and (4) statistical moments, other than mean values of biochemical constituents, significantly impacted C/N ratio estimates and the reliability of these estimates, both of which were positively correlated ($r = 0.64$, $p < 0.001$).

Keywords: Artificial neural networks, C/N ratio, discriminant analysis, predictive models, variance components

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INTRODUCTION

Crop residues vary widely in their chemical composition (Poorter and Villar 1997) and constitute a major source of nutrient inputs to soils in small- (Njunie, Wager, and Luna-Orea 2004) and large-scale (Karlen et al. 1994; Liebig et al. 2002) cropping systems. The quality of these residues regulates their decomposition and the availability of nutrients for subsequent crops in crop rotations. The quality of the crop residue is defined by the organic nitrogen (N) content of the residue and by the carbon (C)/N ratio, which in turn determines the rate of decomposition of the residue and the C/N ratio of microbial biomass (Abiven et al. 2005). Many factors, including the form of the C in plant cells as an energy source, the concentration of other nutrients, and the composition of various secondary compounds (Wang et al. 2004) interact with and impact C/N ratio. In the soil medium, the N dynamics are linked to the C dynamics through the C/N ratio of the various biochemical pools (Hadas, Parkin, and Stahl 1998; Nicolardot, Recous, and Mary 2001).

To predict the fertilizing potential of crop residues, insight into the interrelationships among their biochemical composition and C/N ratio is required (Grant, Peterson, and Campbell 2002). The C/N ratio is frequently used (Cortez et al. 2007) as a quality index of crop residues and their decomposition without taking into consideration the large within-crop variation and co-variation of biochemical constituents. However, this simple index is often misinterpreted (Nicolardot, Recous, and Mary 2001; Flavel and Murphy 2006) as a causal factor, but the gross biochemical composition and the spatial arrangement of constituents in plant tissues are more likely to be the determining factors (Magid, Luxhøi, and Lysede 2004; Jensen et al. 2005).

The literature is replete with arguments (e.g., Quemada and Cabrera 1995; Ruffo and Bollero 2003; Jensen et al. 2005) about the efficacy of the C/N ratio as an indicator of N mineralization in crop residues and whether there is a single critical C/N ratio at which N is released and becomes available for plant use. The C/N ratio of crop residues depends on a number of factors including the plant species, crop genotype, length of the growing season, soil fertility, and environmental conditions (Quemada 2004; Trinsoutrot et al. 2000). For example, C/N ratio, cellulose, and lignin increase with plant age, whereas soluble carbohydrates decrease (Nicolardot, Recous, and Mary 2001). Consequently, younger crop residues have smaller C/N ratios and decompose faster than mature residues.

The biochemical composition (i.e., structural and nonstructural carbohydrates) of crop residues is an important factor in determining C/N ratio and, consequently, the rate of residue decomposition (Hadas

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et al. 2004). Large variability exists in biochemical composition between plants of the same species and between different organs of the same plant, depending on growth conditions (Poorter and Villar 1997) and on morphology and physical characteristics of the plant tissues (Abiven and Recous 2006). Crop plants and plant parts with different chemical composition would be expected to have different C/N ratios and to show different mineralization kinetics (Quemada and Cabrera 1995). We reported on the allocation, associations, and ratios of fixed-C biochemicals in roots, stems, and leaves of two traditional and three alternative crops as candidates in more diverse crop rotations than the current corn–soybean rotation (Johnson, Barbour, and Weyers 2007). Since the residues of these crops are basically composed of similar biochemical constituents but differ in their proportions and level of variation, we hypothesized that statistical moments of biochemical constituents, other than mean values (e.g., minimum, maximum, variance, etc.) can impact C/N ratio prediction when used in multivariate models that account for multicolinearity. The objectives of this study were to (1) identify sources of variation in C/N and quantify their impact on its estimation, (2) build calibration and validation models of C/N ratio in two traditional (corn and soybean) and three alternative (alfalfa, cuphea, and switchgrass) crops and their organs with large variation in biochemical constituents, and (3) identify which statistical moments of these biochemical constituents have the largest impact on predicting C/N ratio.

MATERIALS AND METHODS

The data used to build partial least squares (PLS) calibration and validation models and to discriminate among leaves, stems, and roots of two traditional [corn (*Zea mays* L.), soybean (*Glycine max* L. Merr.)], and three alternative [alfalfa (*Medicago sativa* L.), cuphea germplasm selection (*Cuphea lanceolata* × *Cuphea viscosissima*), and switchgrass (*Panicum virgatum* L.)] crops were derived from biochemical constituents of their leaves, stems, and roots (Johnson, Barbour, and Weyers 2007). Each PLS model was based on four estimates of eight biochemical constituents in leaves, stems, and roots of mature plants sampled from a field experiment.

A data matrix of raw data on a total of 60 samples and eight biochemical constituents (i.e., glucose, fructose, sucrose, starch, cellulose, hemicellulose, acid-insoluble lignin, and acid-soluble lignin), in addition to C, N, and C/N ratio, was used to carry out statistical analyses and to generate a new matrix comprised of mean, minimum, maximum, range, standard deviation, variance, skewness, and kurtosis of each biochemical constituent. The new data matrix was used in artificial neural network analyses (ANN) and sensitivity analyses as described.

Statistical Analyses

Total variance in C, N, and C/N ratio explained by differences among crops, among organs, and among organs within crops, using an orthogonal sum of squares method in a general linear model (GLM), was calculated and tested for significance. A whole model R^2 was calculated for each variable and was partitioned according to its sources of variation (Hair et al. 1998).

C/N Prediction and Validation Models

The partial least squares (PLS) regression option in the nonlinear iterative partial least squares (NIPALS) algorithm (Esbensen 2005) was used on the raw data to construct a set of components that account for as much variation as possible while modeling the biochemical constituents' data. The PLS is an extension of multiple linear regression in the form Y = XB + E, where Y is an "n" cases by "m" variables response matrix, X is an "n" cases by "p" variables predictor matrix, B is a "p × m" regression coefficient (β) matrix, and E is an error term for the model that has the same dimensions as Y. The PLS regression employs rotations to overcome the problem of high-dimensional, correlated data and rotates both the independent and dependent variables to maximize predictive power. The PLS1 option in the Unscrambler software (v 9.7, Camo ASA, 2007) was used for creating models to predict C/N ratio as a function of C, N, or C + N and as a function of eight biochemical constituents in the five crops and the three plant organs. The models developed in this analysis were cross-validated by successively leaving out data one at a time, and a model was built using the remaining data points; then the model created was used to predict C/N ratio. The root mean squares error (RMSE) was used to compare the prediction and validation errors of different PLS regression models and was based on the differences between the predicted and actual values, after all the samples have been held out once. RMSE was calculated as

$$\mathrm{RMSE} = \sqrt{\sum_{i=1}^{n} (\hat{y}_i - y_i)^2 \Big/ n}$$

where $\hat{y}_i$ and y_i are predicted and measured Y and n is the number of samples. PLS was implemented by the Unscrambler v 9.7 (Camo ASA, 2007) software.

Variation between Plant Organs

Canonical discriminant analysis (CDA), a combination of principal components and canonical correlation analyses, was used on the raw data

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to derive canonical variables that contain the largest possible multiple correlation with each plant organ (i.e., leaves, stems, and roots) and that best summarize between-organs variation. The differentiation of leaves, stems, and roots was based on the correlation among the dependent variables (i.e., biochemical constituents) with the independent variable (i.e., plant organs). The differences between mean values of the canonical variables (i.e., group centroids) is the squared Mahalanobis distance D^2 and is calculated as

$$D^2 = (\overline{X}_1 - \overline{X}_2) S^{-1} (\overline{X}_1 - \overline{X}_2)$$

where S^{-1} is the inverse of the pooled sample variance–covariance matrix and $\overline{X}_1$ and $\overline{X}_2$ are the respective vectors of measurements of organs 1 and 2 (e.g., leaves and stems). The R^2 values in CDA were used to identify the traits that most significantly contributed to the discrimination among plant organs (StatSoft Inc. 2007b).

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Sensitivity Analyses of C/N Ratio

The impact of statistical moments of biochemical constituents on C/N ratio and RMSE estimates was studied using feed-forward, back-propagation artificial neural network (ANN) module in Statistica 8.0 (StatSoft Inc. 2007a) using a data matrix composed of all statistical moments described (see Materials and Methods). Because of data size limitations (Statsoft Inc. 2007b), ANN analysis was performed on the whole data set for all crops combined. The algorithm is based on supervised learning; the learning phase consists of adjusting the weights of the network connections by feeding the learning data set many times. After training with a learning data set, the network was fed with a validation data set, and the proportion of correct predictions was used to assess the reliability of the network model. ANN models were subjected to sensitivity analysis to evaluate the relative importance of each independent variable in explaining variation in C/N ratio and in RMSE estimates. In this analysis, each predictor was treated in turn as if it was not available in the ANN model, and the average value of that predictor was used. A sensitivity ratio was calculated by dividing the total ANN error when the predictor was treated as "not available" by the total ANN error when the actual value of the predictor was used. If the sensitivity ratio is >1.0, then the predictor made an important contribution to C/N ratio; the greater the ratio, the more important the predictor. Additionally, we calculated the correlation coefficient (r value), a ratio between the standard deviation (SD ratio) of original and model data, the absolute error mean, and RMSE; higher r values and lower SD ratio, absolute error mean, and RMSE values of the test sample are

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indicators of better model performance (StatSoft Inc. 2007b). Sensitivity analyses were performed by generating response curves for each predictor to study its impact on C/N ratio or RMSE, while all other predictors were set at their mean value.

RESULTS

Sources of Variation in C/N Ratio

Total variances in C, N, and C/N ratio were partitioned into their components according to three sources of variation: among crops, among organs, and among organs within crops (Table 1). These sources of variation explained 91.0, 97.0, and 87.0% of total variance in C, N, and C/N, respectively, as indicated by the adjusted R^2 values. When total variance for each dependent variable was tested for significance, all three sources of variation accounted for significant portions of variances in N and C/N ratio, whereas only differences among organs within crops accounted for a large and significant (70.9%) portion of variance in C.

Variation between Plant Organs

Crops and organs differed significantly in their C/N ratio (overall mean = 48.0, CV 44.6%; Table 2). Alfalfa and cuphea had the smallest and switchgrass had the largest C/N ratios, whereas leaves had the smallest C/N ratio, followed by roots and stems. The whole-model R^2 values of the validation models were smaller, and the RMSE estimates were larger, than the values of their respective calibration models. Coefficients of the calibration and validation models in the PLS regression analyses to predict C/N ratios based on C, N, or C + N contents exhibited large differences between crops and between organs. Strong relationships were

Table 1. Variance explained (adjusted R^2) and percentage variance in N, C, and C/N ratio accounted for by differences among crops (alfalfa, corn, cuphea, soybean, and switchgrass), among organs (leaves, stems, and roots), and among organs within crops.

Variable	Adjusted R^2	Variance (%)		
		Crops	Organs	Organ (crops)
Carbon	91.0**	19.7	1.0	70.9**
Nitrogen	97.0**	53.3**	33.7**	10.3**
C/N ratio	87.0**	35.0**	35.0**	14.4**

**Significant at $p < 0.001$.

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Table 2. Coefficients and test statistics of partial least squares (PLS) regression models to predict C/N ratio in five (alfalfa, corn, cuphea, soybean, and switchgrass) crops and their organs (stems, leaves, and roots) using N, C, or N + C percentages as independent variables (X)

Crops/organs	C/N ratio	X	Calibration model				Validation model			
			Intercept	Slope	R^2 value	RMSE	Intercept	Slope	R^2 value	RMSE
All	48.0	N	21.8	0.55	54.0	22.9	21.4	0.54	52.0	24.1
		C	40.5	0.16	16.0	31.2	41.0	0.14	13.0	32.3
		N + C			26.0				22.0	
Crops										
Alfalfa	17.2d[a]	N	0.33	0.98	98.0	0.7	0.3	0.98	97.0	0.8
		C	12.5	0.27	27.0	4.2	14.7	0.17	2.0	5.3
		N + C			93.0				92.0	
Corn	49.6bc	N	12.3	0.75	75.0	11.6	13.9	0.69	67.0	14.4
		C	28.7	0.42	42.0	17.7	31.8	0.33	32.0	20.9
		N + C			43.0				33.0	
Cuphea	31.4cd	N	2.9	0.90	90.0	7.3	2.1	0.91	85.0	5.9
		C	10.9	0.63	63.0	8.4	10.0	0.63	54.0	10.4
		N + C			68.0				58.0	
Soybean	66.2ab	N	9.5	0.86	86.0	12.9	8.9	0.85	82.0	14.9
		C	21.4	0.68	68.0	18.7	25.1	0.62	63.0	21.8
		N + C			71.0				76.0	
Switchgrass	75.8a	N	13.9	0.82	82.0	16.4	12.0	0.82	73.0	21.8
		C	71.6	0.05	5.0	37.0	85.0	−0.09	0.0	44.0
		N + C			45.0				31.0	

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Table 2. Continued

Crops/organs	C/N ratio	X	Calibration model				Validation model			
			Intercept	Slope	R^2 value	RMSE	Intercept	Slope	R^2 value	RMSE
Organs										
Stems	74.5a[b]	N	17.5	0.77	77.0	18.7	17.1	0.76	75.0	20.5
		C			0.0				0.0	
		N + C			64.0				60.0	
Leaves	24.4c	N	5.5	0.78	78.0	5.32	5.4	0.77	75.0	5.9
		C			0.0				0.0	
		N + C			71.0				59.0	
Roots	45.3b	N	14.9	0.67	67.0	13.2	15.6	0.65	62.0	14.8
		C			0.0				0.0	
		N + C			19.0				12.0	

[a]C/N ratio means of crops followed by the same letter do not differ significantly at the 5% level of probability (Tukey's HSD).
[b]C/N ratio means of organs followed by the same letter do not differ significantly at the 5% level of probability (Tukey's HSD).

found between the intercept and RMSE estimates in the calibration ($r = 0.91$; $p < 0.01$) and validation ($r = 0.89$; $p < 0.01$) models.

C/N Prediction and Validation Models

Variation in N, but not in C or N + C, content explained the greatest variance ($R^2 > 73.0$ for crops and >62.0 for organs) in C/N ratios in all crops combined, in individual crops, and in crop organs (Table 2). The reliability of estimation when N content was used as a predictor was large for alfalfa, cuphea, and soybean and moderate for corn and switchgrass. The R^2 values in the validation models were 97.0, 67.0, 85.0, 82.0, and 73.0 for alfalfa, corn, cuphea, soybean, and switchgrass, respectively, whereas the respective RMSE estimates were 0.8, 14.4, 5.9, 14.9, and 21.8. Nevertheless, R^2 values of validation models for stems (75.0), leaves (75.0), and roots (62.0) were very close to their respective estimates in the calibration models (77.0, 78.0, and 67.0, respectively).

Variation in C content explained no ($R^2 = 2.0$, in alfalfa, 0.0 in switchgrass, stems, leaves, and roots), small ($R^2 = 32.0$ in corn), or moderate ($R^2 = 54.0$ in cuphea and 63.0 in soybean) portions of total variance in C/N ratios; however, when N + C values were used as predictors, the R^2 values were intermediate between those predicted by N or C only. In addition, RMSE estimates for the C-based validation models were much larger than the respective values for N-based validation models.

Coefficients of the calibration and validation models in the PLS regression analyses to predict C/N ratios in all five crops and their organs based on their biochemical constituents (Table 3) exhibited large differences between crops and between organs. When compared with the N-based PLS models, slightly smaller, albeit significant, relationships were found between the intercept and RMSE in the calibration ($r = 0.77$; $p < 0.05$) and validation ($r = 0.75$; $p < 0.05$) models when all biochemical constituents were used as predictors. Reliability of C/N ratio estimation, as quantified by the validation R^2 values, was large for alfalfa (85.0) and cuphea (82.0), intermediate for corn (61.0), and small for soybean (52.0) and switchgrass (46.0). Calibration and validation R^2 values were similar in magnitude when C/N ratios in leaves, stems, and roots were estimated using all biochemical constituents. However, when biochemical constituents or C/N ratios in stems and leaves were used to estimate C/N in roots, the R^2 values [roots (1)–roots (6); Table 3] were highly variable and dropped from 61.0 (when biochemicals in all three organs were used as predictors) to 20.0 (when only biochemicals in leaves were used).

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Table 3. Coefficients and test statistics of partial least square (PLS) regression models to predict C/N in five (alfalfa, corn, cuphea, soybean and switchgrass) crops and their organs (stems, leaves and roots) using eight (glucose, fructose, sucrose, starch, cellulose, hemicellulose, acid soluble lignin, and acid insoluble lignin) biochemical constituents

Crop/organ	Calibration model				Validation model			
	Intercept	Slope	R^2 value	RMSE	Intercept	Slope	R^2 value	RMSE
All crops	20.4	0.55	55.0	19.0	22.3	0.47	52.0	20.9
Alfalfa	1.93	0.88	89.0	1.66	2.64	0.84	85.0	1.87
Corn	12.3	0.75	75.0	23.2	15.6	0.68	61.0	29.3
Cuphea	1.06	0.87	88.0	1.61	1.94	0.82	82.0	2.10
Soybean	25.7	0.66	62.0	20.2	32.3	0.57	52.0	23.0
Switchgrass	28.9	0.62	59.0	23.9	35.0	0.49	46.0	29.0
Leaves	9.96	0.59	59.0	7.7	11.1	0.55	49.0	8.0
Stems	30.8	0.54	54.0	20.3	31.8	0.53	54.0	21.0
Roots	14.1	0.68	68.0	12.6	18.4	0.59	54.0	16.4
Roots (1)[a]	13.3	0.70	71.0	16.6	14.9	0.66	61.0	19.1
Roots (2)	13.2	0.71	71.0	17.4	15.0	0.66	61.0	19.6
Roots (3)	14.2	0.69	78.0	16.7	16.0	0.63	58.0	19.2
Roots (4)	30.1	0.33	33.0	18.4	33.7	0.25	20.0	20.5
Roots (5)	23.6	0.48	57.0	16.3	26.3	0.43	34.0	18.8
Roots (6)	24.4	0.46	46.0	16.6	27.1	0.42	28.0	19.2

[a]Independent variables are biochemical constituents: (1) in leaves, stems, and roots; (2) in stems and leaves; (3) in stems; (4) in leaves; (5) only C/N ratio in stems and leaves; and (6) only C/N ratio in stems.

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Six calibration and validation models were constructed to estimate C/N ratio in roots. A relatively better prediction of C/N in the roots was achieved when biochemical constituents in all organs (R^2 = 61.0), in stems and leaves (R^2 = 61.0), or in roots (R^2 = 54.0) were used as predictors, as compared to predictions using the remaining models (Table 3). Biochemical constituents in leaves contributed very little to predicting C/N ratio in roots, whether alone (R^2 = 20.0), or in combination with stems (R^2 = 61.0), as compared to using biochemicals in stems alone (R^2 = 58.0). However, C/N ratios in both stems and leaves, and in stems alone, explained only 34.0 and 28.0% of variation in C/N ratio in the roots, respectively.

Associations between individual biochemicals and PLSCs (r values) ranged from −0.5 to +0.5 on PLSC1 and from −0.6 to +0.8 on PLSC2 (Figures 1–5). The PLS regression analyses partitioned the eight biochemical constituents along the first two components and explained large portions ($R^2 > 0.78$) of total variances in C/N ratios in each crop. The CDA resulted in full separation of leaves, stems, and roots of each crop. Stems and roots were invariably separated from leaves on CAN1, with large (>82.0) R^2 values, whereas stems and roots were separated from each other along CAN2 but with smaller (4.0–18.0) R^2 values. In addition, all D^2 values of leaves, stems, and roots of each crop, based on biochemical constituents, were significant (largest $p < 0.008$). Correlation coefficients between single biochemical constituents and the first two PLSCs and covariations (i.e., r values on positive and negative sides of PLSC1 and PLSC2) among these constituents differed among crops and contributed to full discrimination between plant organs within each crop.

In alfalfa, a C_4 perennial forage legume crop, the first two PLSCs accounted for 77% of total variance in C/N ratio (Figure 1A), whereas CAN1 and CAN2 explained 83 and 17% of total variance, respectively, and fully discriminated among its stems, roots, and leaves (Figure 1B). The PLSCs separated the eight biochemical constituents into three groups. The first (glucose, fructose, acid-insoluble lignin, and cellulose), second (hemicellulose, sucrose, and starch), and third (acid-soluble lignin) were associated with stems, roots, and leaves, respectively. Monosaccharides were totally separated from starch and sucrose with large covariances on PLSC1, whereas cellulose and hemicellulose were closely associated on PLSC1 with a small covariance. Both acid-soluble and acid-insoluble lignin had significant R^2 values in the discriminant analysis between organs and were associated negatively and positively on PLSC1, respectively. Loadings of biochemical constituents on PLSC1 and PLSC2 and their R^2 values, derived from CDA in Figure 1A, suggest that larger values of acid-insoluble lignin, starch, sucrose, and glucose, in decreasing order, and smaller values of acid-insoluble lignin, contributed to full discrimination of alfalfa stems and roots from its leaves. Similarly, biochemicals with

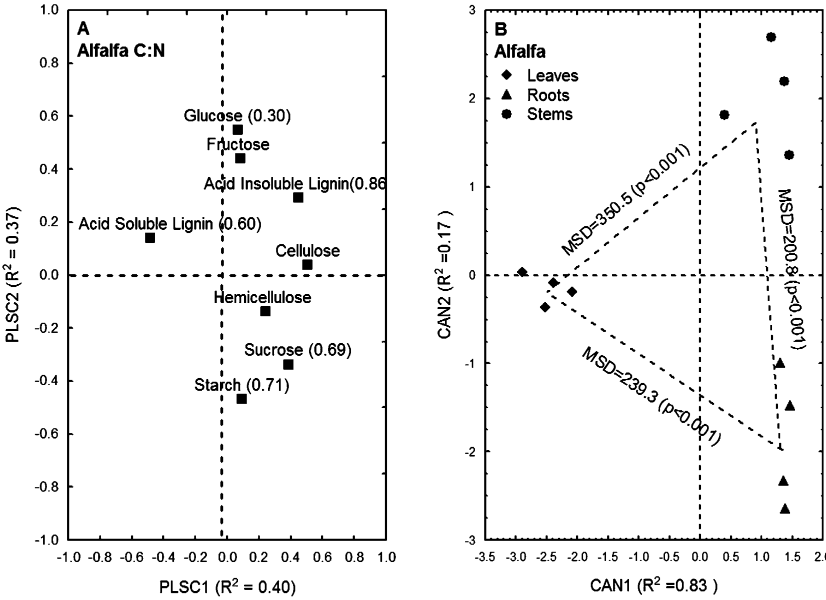

Figure 1. Loadings, significant R^2 values of biochemical constituents, and amount of variation in C/N ratio accounted for by the first two partial least squares (PLS) components (A); and discrimination, squared Mahalanobis distances (D^2), and mean separation among C/N ratio estimates in leaves, stems, and roots of alfalfa based on their biochemical constituents (B).

significant R^2 values and with either positive or negative loadings on PLSC2 contributed to full discrimination between stems and roots. Only three biochemicals (i.e., cellulose, hemicellulose, and fructose, with nonsignificant R^2 values) did not contribute to the multivariate discrimination among alfalfa organs. Stems and roots were separated by the smallest distance ($D^2 = 200.8$; $p < 0.001$), followed by an intermediate distance between stems and leaves ($D^2 = 239.3$; $p < 0.001$), and then by the largest distance ($D^2 = 350.5$; $p < 0.001$) between leaves and stems.

In corn (Figure 2), an annual C_4 cereal crop, PLS analyses resulted in a slightly different multivariate display of the association between biochemical constituents on the first two PLSCs, the proportion of explained variance in its C/N ratio, and discrimination among its organs. The first two PLSCs accounted for 76% of total variance in C/N ratio (Figure 2A), with sucrose, cellulose, and hemicellulose, in decreasing order, significantly contributing toward full discrimination among leaves, stems, and roots; the R^2 value for starch (7.0) was not significant. More than half ($R^2 = 55\%$) of total variance in biochemical constituents was accounted for by PLSC1, with positive loadings of all biochemical constituents except acid-soluble lignin. PLSC2 accounted for 21% of the

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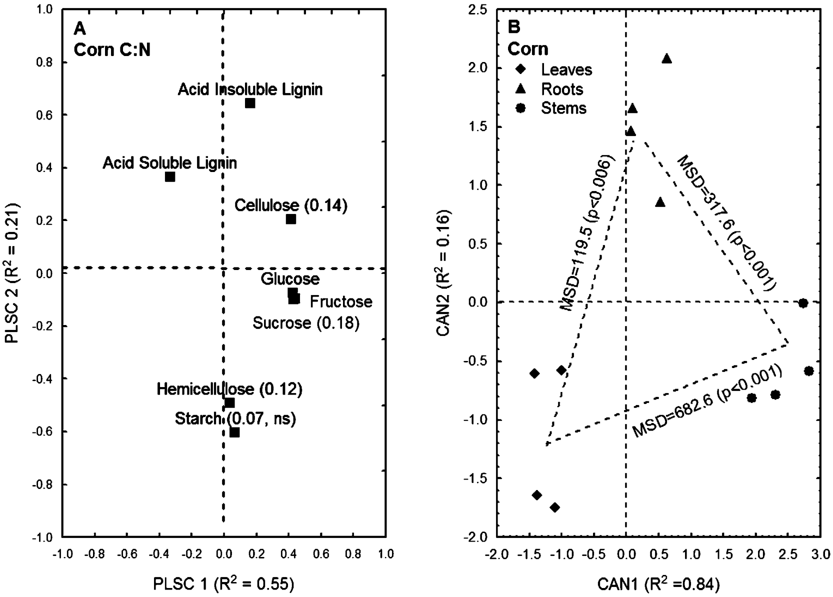

Figure 2. Loadings, significant R^2 values of biochemical constituents, and amount of variation in C/N ratio accounted for by the first two PLS components (A); and discrimination, squared Mahalanobis distances (D^2), and mean separation among C/N ratio estimates in leaves, stems, and roots of corn based on their biochemical constituents (B).

variance in biochemical constituents and contributed to full discrimination between roots and other organs. The significant R^2 values associated with cellulose, hemicellulose, and sucrose were small in magnitude (12.0–18.0). Soluble sugars were clustered together with small covariation on PLSC1, whereas most covariation was displayed by acid-soluble and acid-insoluble lignin. The D^2 distances (Figure 2B) between corn organs were all significant, with leaves and roots being separated by the smallest distance (D^2 = 119.5; p < 0.006), followed by a medium distance separating stems and roots (D^2 = 317.6; p < 0.001), and then by the largest distance (D^2 = 682.6; p < 0.001) separating stems and leaves.

Cuphea (Figure 3A) represents a unique case of a semidomesticated C_3 annual plant; it is being developed as a potential oilseed crop with large lipid content in its seed and, to some extent, in its leaves. The first two PLSCs accounted for 53 and 15% of total variance in C/N ratio, respectively (Figure 3A). Most biochemical constituents had large loadings on PLSC1, except sucrose and hemicellulose. Acid-soluble and acid-insoluble lignin, followed by starch and cellulose, with large and significant ($p < 0.05$) R^2 values of 98.0, 97.0, 77.0, and 52.0, respectively, were the most discriminating biochemical constituents among cuphea

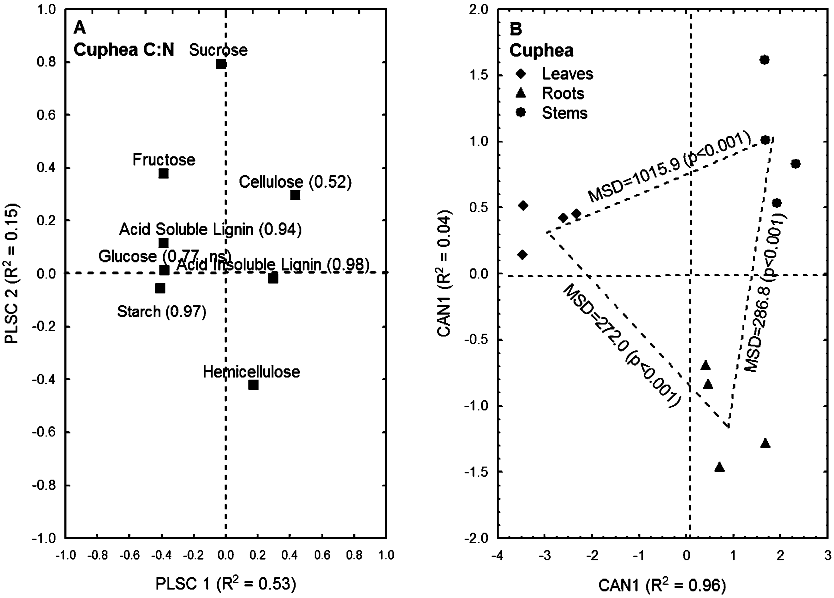

Figure 3. Loadings, significant R^2 values of biochemical constituents, and amount of variation in C/N ratio accounted for by the first two PLS components (A); and discrimination, squared Mahalanobis distances (D^2), and mean separation among C/N ratio estimates in leaves, stems, and roots of cuphea based on their biochemical constituents (B).

organs, specifically among its leaves and both of its stems and roots. Covariation was large between acid-soluble and acid-insoluble lignin, followed by covariation between cellulose and hemicellulose. Most variation among cuphea organs (R^2 = 96.0) was accounted for by CAN1 (Figure 3B) and was due to differences among plant organs in concentrations of biochemical constituents with large positive and negative loadings on PLSC1. CAN1 totally separated leaves from stems and roots of cuphea, whereas CAN2 separated roots from both stems and leaves, albeit with very small R^2 (4.0) value (Figure 3B). The D^2 separating stems and roots (D^2 = 286.8; p < 0.001) and the one separating roots and leaves (D^2 = 272.0; p < 0.001) were much smaller than the distance separating leaves and stems (D^2 = 1015.9; p < 0.001).

Almost equal proportions of C/N ratio variance were accounted for by PLSC1 (36.0) and PLSC2 (32.0) in soybean (Figure 4A), an annual C_3 traditional legume crop. Soluble sugars and hemicellulose were the only biochemical constituents that did not contribute significantly to the discrimination between soybean leaves, stems, and roots. Cellulose and starch had the largest R^2 values (97.0), followed, in decreasing order, by acid-insoluble lignin (74.0) and acid-soluble lignin (45.0). Soluble sugars

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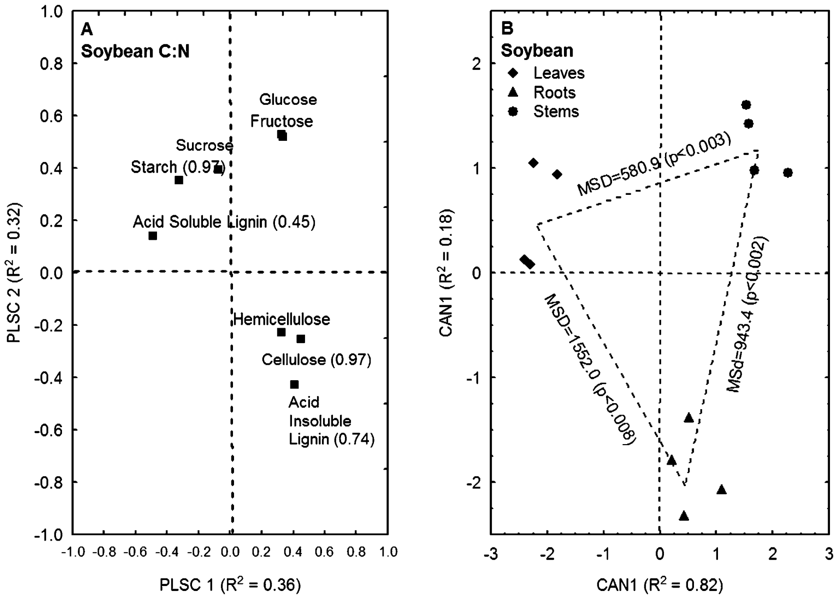

Figure 4. Loadings, significant R^2 values of biochemical constituents, and amount of variation in C/N ratio accounted for by the first two PLS components (A); and discrimination, squared Mahalanobis distances (D^2), and mean separation among C/N ratio estimates in leaves, stems, and roots of soybean based on their biochemical constituents (B).

and starch, similar to cellulose and hemicellulose, had small covariances. Acid-soluble and acid-insoluble lignin had large covariances as a result of large opposite loadings on both PLSCs. The CAN1 accounted for 82.0% of total variance between soybean organs and separated leaves from stems and roots based on differences in cellulose and acid insoluble lignin, on one hand, and starch and acid soluble lignin, on the other. The separation between roots and each of stems and leaves on CAN2 was smaller in magnitude ($R^2 = 18.0$). The D^2 values separating soybean organs from each other were the largest as compared to other crops (Figure 4B). The smallest distance ($D^2 = 580.9$; $p < 0.003$) separated leaves from stems, the intermediate distance ($D^2 = 943.4$; $p < 0.002$) separated stems from roots, and the largest distance ($D^2 = 1552.0$; $p < 0.008$) separated leaves from roots.

Finally, in switchgrass, a C_4 perennial biomass crop, PLSC1 and PLSC2 explained 44.0 and 28.0% of total variance in C/N ratio and separated acid-soluble lignin and acid-insoluble lignin from the remaining biochemical constituents, respectively (Figure 5A). Most biochemical constituents, except sucrose and cellulose, contributed to the explained variance in C/N ratio on PLSC1, whereas sucrose and acid-insoluble lignin

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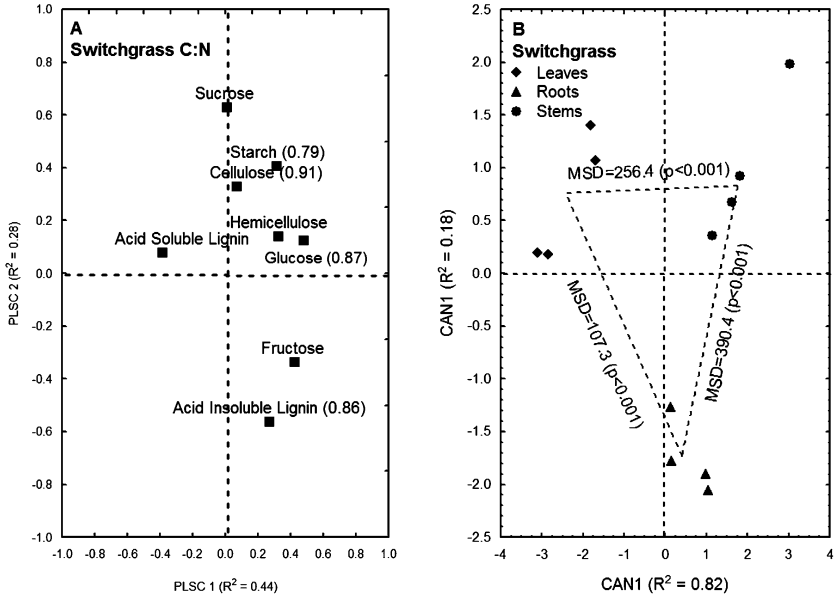

Figure 5. Loadings, significant R^2 values of biochemical constituents, and amount of variation in C/N ratio accounted for by the first two PLS components (A); and discrimination, squared Mahalanobis distances (D^2), and mean separation among C/N ratio estimates in leaves, stems, and roots of switchgrass based on their biochemical constituents (B).

explained most of the variance in C/N ratio on PLSC2. Four biochemical constituents (i.e., cellulose, glucose, acid-insoluble lignin, and starch, in decreasing order) contributed significantly ($R^2 > 79.0$; $p < 0.05$) to the discrimination among leaves, stems, and roots of switchgrass. Starch and soluble sugars were dispersed along PLSC2, whereas cellulose and hemicellulose had a small covariance as compared to acid-soluble and acid-insoluble lignin. CAN1 and CAN2 explained 82.0 and 18.0% of total variance among stems, roots, and leaves, respectively (Figure 5B); however, the D^2 values separating these plant organs were among the smallest in this study. The smallest distance (D^2 = 107.3; $p < 0.001$) separated leaves from roots on both PLSCs, the intermediate distance (D^2 = 256.4; $p < 0.001$) separated stems from leaves on PLSC1, and the largest distance (D^2 = 390.4; $p < 0.001$) separated stems from roots on PLSC2.

Reliability of C/N Ratio and RMSE Estimation

A validation PLS model for all crops and their organs revealed a positive and significant ($r = 0.64$; $p < 0.001$) relationship between C/N ratio and

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RMSE estimates. Two crops (alfalfa and cuphea) and two plant organs (leaves and roots) had below-average values of both. Switchgrass had the largest C/N ratio but below-average RMSE, whereas corn, soybean, and stems had above-average values for both. Reliability of ANN analysis was tested by comparing several statistics of the training and testing samples (Table 4). Mean C/N ratio was smaller for the testing sample (49.3) as compared to the training sample (52.5). However, all other statistics of the testing sample were slightly larger than those for the training sample. Consequently the R^2 for the testing sample (68.0) was smaller than the respective value for the training sample (92.0). A similar trend was observed in the RMSE statistics for training and testing samples, except that SD of the testing sample was smaller than the one for the training sample.

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Sensitivity Analyses of C/N Ratio and RMSE Estimates

Sensitivity analyses identified six independent variables out of the 64 statistics calculated for the eight biochemical constituents as important in determining C/N ratio (Figure 6); these were maximum values of cellulose, starch, acid-insoluble lignin, hemicellulose, and glucose and minimum values of starch. The relationships between these variables and C/N ratio were linear positive (maximum cellulose, maximum hemicellulose, and minimum starch), nonlinear positive (maximum glucose), and linear negative (maximum acid-insoluble lignin). The ratio values derived from the ANN sensitivity analyses indicated that maximum cellulose and maximum glucose had the most and least impact on C/N ratio estimates, respectively. Similarly, sensitivity analyses identified six independent variables out of the 64 statistics calculated for the eight biochemical constituents as important in determining RMSE (Figure 7); these were maximum values of glucose and acid-soluble lignin; SD values of cellulose, acid-soluble, and acid-insoluble lignin; and mean values of starch contents. The relationships of these variables and

Table 4. Neural network test statistics for C/N ratio and RMSE estimates of training and testing samples derived from biochemical analysis of leaves, stems, and roots of five (alfalfa, corn, cuphea, soybean, and switchgrass) crops in a 500-day decomposition study.

Dependent variable	Sample	Neural network statistics					
		Mean	SD	SD ratio	Absolute error mean	R^2	RMSE
C/N	Training	52.5	21.8	0.012	0.12	92.0	6.2
	Testing	49.3	24.8	0.017	0.16	68.0	10.3
RMSE	Training	15.5	11.4	0.003	0.03	88.0	
	Testing	14.0	7.9	0.004	0.05	64.0	

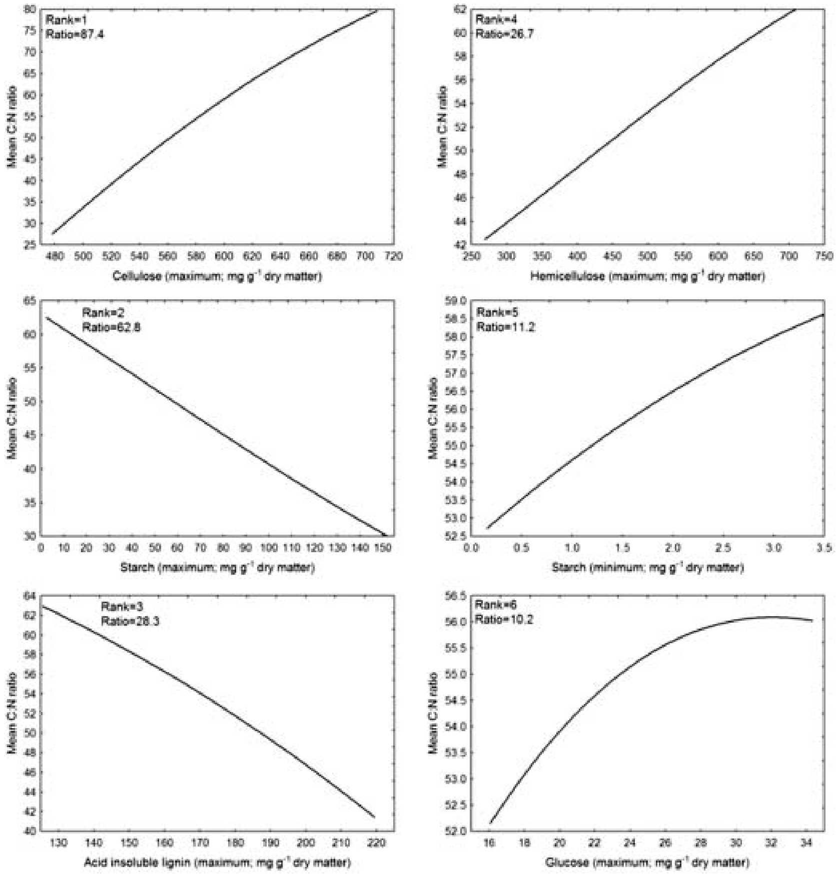

Figure 6. Sensitivity analyses of the most important biochemical constituents in predicting C/N ratio in leaves, stems, and roots of five (alfalfa, corn, cuphea, soybean, and switchgrass) crops.

RMSE were linear positive (maximum glucose and cellulose SD), nonlinear positive (acid-insoluble lignin SD), linear negative (acid-soluble lignin SD), and nonlinear negative (mean starch and maximum acid-soluble lignin). The ratios derived from the ANN sensitivity analyses indicated that maximum glucose and SD of acid-soluble lignin had the largest and smallest impact on RMSE estimates, respectively.

DISCUSSION

The long-term objective of studying the composition and decomposition of this diverse set of crops is to model optimal crop sequences for nutrient

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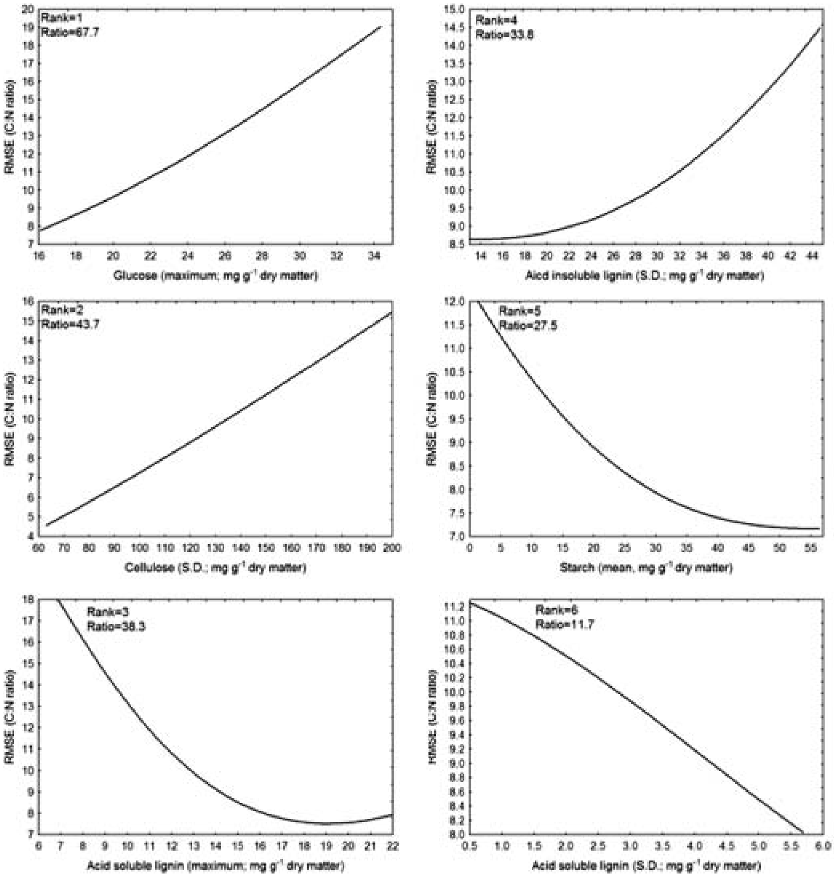

Figure 7. Sensitivity analyses of the most important biochemical constituents in predicting RMSE of C/N ratio in leaves, stems, and roots of five (alfalfa, corn, cuphea, soybean, and switchgrass) crops.

cycling and for the production of biomass for bioenergy. The five crops used in this study were selected to encompass as wide a quality range as possible with respect to C/N ratio (average 17.2–75.8) and distribution of C and N in biochemical fractions of their leaves, stems, and roots (Johnson, Barbour, and Weyers 2007), especially when compared with C/N ratios and biochemical fractions of 14 diverse crops (average 9.4–46.5; Chaves et al. 2004), or plant communities sampled from natural habitats (average 30.5–71.14; Cortez et al. 2007).

The small portion (14.4%) of total variance in C/N ratio due to organs within crops as compared to the relatively larger portions (35.0%) due to each of crops and organs provides a guideline as to whether residues of single or multiple crops should be used for nutrient cycling or

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as a source of biomass for bioenergy production. Plant material with large C/N ratio (e.g., switchgrass) is considered to be energy-rich material (Cortez et al. 2007), whereas plant material with small C/N ratio (e.g., alfalfa and cuphea) may contribute to nutrient cycling and soil fertility (Chaves et al. 2004).

Simple PLS models were developed to predict and validate C/N ratio in crops and organs with different levels of goodness of fit and accuracy (i.e., R^2 and RMSE, respectively). Whether based on variation in N but not C or N + C (Table 2) or on variation in biochemical constituents (Table 3), C/N models reflect sizable differences in N content and biochemical composition among crops and among organs. By definition, C is positively and N is negatively correlated with C/N ratio. Variation in C concentration in plants generally ranges between 400 and 500 $mg\,g^{-1}$, whereas N may vary more than fivefold (Jensen et al. 2005); consequently, C/N ratio may vary more than 20-fold. On average, N varied from 4.36 to 44.0 and C from 343 to 468 $g\,kg^{-1}$ plant material in these crops (Johnson, Barbour, and Weyers 2007). Hence, the strong relationship between N content and biochemical composition of crop residues, which itself is usually related to the nature, age, and growing conditions of the crops (Nicolardot, Recous, and Mary 2001; Cortez et al. 2007) and that C/N ratio is the best predictor of potential N that can be mineralized from a crop residue (Chaves et al. 2004). Predictions of N-based validation models for crops and organs (Table 2) are in agreement with results obtained for a wide range of grain (Nicolardot, Recous, and Mary 2001), oil crops (Trinsoutrot et al. 2002), and plants sampled from natural habitats (Cortez et al. 2007).

Validation models based on all biochemical constituents were crop-specific (Table 3); their R^2 and C/N ratios were inversely related ($r = -0.98$, $p < 0.05$, $n = 5$), suggesting that small C/N ratios can be predicted more accurately than large ones. This finding is supported indirectly by the substantially smaller RMSE value for leaves (7.7), which have a smaller C/N ratio than roots (24.9), in modeling mineralization of C and N from residues of diverse plants (Abiven et al. 2005). Validation models for C/N ratio in roots (Table 3) confirm the close biochemical relationship between stems and roots (Poorter and Villar 1997). Biochemical composition of stems alone explained 58% of variation in C/N estimates in roots, notwithstanding C/N differences among crops as implied by the small R^2 value (28.0) when C/N in stems was used to predict C/N in roots, and by the results of C/N mean separation among organs in each crop (Figures 1–5). In addition, we calculated ratios of concentrations of biochemicals in stems and roots relative to those of leaves for the same crops (Johnson, Barbour, and Weyers 2007) and found that shoots/leaves ratios of glucose, fructose, cellulose, hemicellulose, and acid-insoluble lignin, as well as roots/leaves ratios of sucrose, cellulose, hemicellulose,

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and acid-insoluble lignin, were larger than and significantly ($p < 0.05$) different from 1.0. However, the remaining shoots/leaves and roots/leaves ratios of starch and acid-soluble lignin were less than and significantly ($p < 0.05$) different from 1.0, whereas the shoots/leaves and roots/leaves C/N ratios were significantly ($p < 0.05$) different from 1 (3.0 and 1.9, respectively).

Covariation in biochemical composition and its impact on C/N ratio estimates (Figures 1–5) may reflect inherent differences between these crop plants; however, it may be environmentally induced (Poorter and Villar 1997) or impacted by management (Carpenter-Boggs et al. 2000; Dubeux et al. 2006). Differences in C/N ratios between stems and roots, although significant except for alfalfa, were small, especially when compared with those of leaves. This finding supports Poorter and Villar's (1997) suggestion to combine stems and roots for biochemical analyses if time and resources are limited.

The different patterns of covariation among groups of biochemicals and their association with C/N ratios (Figures 1–5) are dependent on which biochemicals represented most of total C in each crop or plant organ as suggested by Jensen et al. (2005) and are supported by the large and significant variance components due to crops and organs within crops (Johnson, Barbour, and Weyers 2007). Acid-insoluble and acid-soluble lignins, representing most of total C, invariably covaried in all crops; the first with strong association with stems and roots, the second with leaves. Covariation of cellulose and hemicellulose was less intense, and both biochemicals were strongly associated with stems and roots. Covariation of soluble sugars and starch displayed no unique pattern. Biochemicals representing most of total C display highly significant, negative correlation coefficients (Poorter and Villar 1997; Jensen et al. 2005).

There was no significant overfitting during the training phase of the ANN as indicated by the R^2 values for C/N and RMSE (92.0 and 88.0, respectively). These values indicate that ANN was slightly better in predicting C/N than RMSE in this data set. However, the respective R^2 values for the validation models (68.0 and 64.0) may reflect the relatively small data set available for validation (StatSoft Inc. 2007). Sensitivity analyses (Figure 6) suggest that biochemicals, where most plant C is assimilated (e.g., cellulose, hemicellulose, acid-insoluble lignin, and acid-soluble lignin), were dominant in and may inflate the estimation of predicting C/N ratio and the accuracy of this prediction (Figure 7). This finding is supported by the findings of Jensen et al. (2005), who showed, for example, that plant samples with less than 200 mg of cellulose and hemicellulose (i.e., holocellulose) C g^{-1} all had low C/N ratios (between 7 and 23) and that the overall correlation between holocellulose C and total C/N ratio was significant (0.67). However, these researchers showed that residue samples with more than 200 mg holocellulose C g^{-1} had highly varying and skewed C/N ratios (18–227). Another supporting statistical evidence was presented by Poorter and Valler (1997), who concluded that C/N ratio estimates for 24 herbaceous species were strongly associated with large values of total structural carbohydrates in stems and roots and with large values of total nonstructural carbohydrates in leaves.

CONCLUSIONS

We developed predictive models that can reliably estimate C/N ratios in stems, leaves, and roots of five diverse crops characterized by large variation in eight biochemical constituents, only three to five of which were adequate to fully discriminate among their organs. Sensitivity analyses identified which statistical moments (e.g., maximum, standard deviation), other than mean values, of these biochemical constituents have the largest impact on estimating C/N ratios and on the reliability of these estimates. Notwithstanding the inconsistent and variable relationships between biochemical constituents and C/N ratios of stems, roots, and leaves of the five crops, stems, on a multivariate scale, were closer to roots than to leaves. Results of this study provide insights into the interrelationships among biochemical composition and C/N ratios necessary for agronomists and farmers to predict the fertilizing potential of crop residues and to design more diverse and viable crop rotations.

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